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'Endotoxin tolerance': TNF-α hyper-reactivity and tubular cytoresistance in a renal cholesterol loading state

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The term 'endotoxin tolerance' defines a state in which prior endotoxin (lipopolysaccharide (LPS)) exposure induces resistance to subsequent LPS attack. However, its characteristics within kidney have not been well defined. Hence, this study tested the impact of LPS 'preconditioning' (LPS-PC; 18 or 72 h earlier) on: (i) selected renal inflammatory mediators (tumor necrosis factor (TNF)-α, interleukin-10 (IL-10), monocyte chemotactic protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), Toll-like receptor 4 (TLR4); protein or mRNA); (ii) cholesterol homeostasis (a stress reactant); and (iii) isolated proximal tubule (PT) vulnerability to hypoxia or membrane cholesterol (cholesterol oxidase/esterase) attack. Two hours post LPS injection, LPS-PC mice manifested reduced plasma TNF-α levels, consistent with systemic LPS tolerance. However, in kidney, paradoxical TNF-α hyper-reactivity (protein/mRNA) to LPS existed, despite normal TLR4 protein levels. PT TNF-α levels paralleled renal cortical results, implying that PTs were involved. LPS-PC also induced: (i) renal cortical iNOS, IL-10 (but not MCP-1) mRNA hyper-reactivity; (ii), PT cholesterol loading, and (iii) cytoresistance to hypoxia and plasma membrane cholesterol attack. A link between cholesterol homeostasis and cell LPS responsiveness was suggested by observations that cholesterol reductions in HK-2 cells (methylcyclodextrin), or reductions in HK-2 membrane fluidity (A2C), blunted LPS-mediated TNF-α/MCP-1 mRNA increases. In sum: (i) systemic LPS tolerance can be associated with renal hyper-responsiveness of selected components within the LPS signaling cascade (e.g., TNF-α, iNOS, IL-10); (ii) PT cytoresistance against hypoxic/membrane injury coexists; and (iii) LPS-induced renal/PT cholesterol accumulation may mechanistically contribute to each of these results.

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In the aftermath of acute endotoxin exposure, animals become relatively resistant to further endotoxin challenges.[1–5] This state, denoted by the term 'endotoxin tolerance', manifests itself by reductions in cytokine generation, suppressed cell-mediated inflammation, and ultimately, decreased tissue damage.[1–5] The mechanism(s) by which animals acquire endotoxin/lipopolysaccharide (LPS) tolerance remain poorly defined. However, downregulation of Toll-like receptor(s) or of their 'downstream' signaling pathways may be partially involved.[1]

Whether the kidney acquires LPS tolerance, and how such tolerance might manifest itself, are unclear at this time. Following LPS exposure, the kidney becomes relatively resistant to nephrotoxin-induced acute renal failure (e.g., Honda *et al.*[6] and Nath *et al.*[7,8]). Whether resistance to ischemic injury also results is controversial.[8,9] In states in which LPS-induced renal cytoprotection against ischemia has been observed,[9] it has been mechanistically linked to a downregulation of systemic inflammatory mediators, secondarily decreasing evolving renal damage.[9,10] Whether LPS tolerance directly protects proximal tubules (PTs) from superimposed adenosine trisphosphate depletion injury has not been defined. Furthermore, the impact of prior LPS exposure on subsequent LPS-initiated inflammatory pathways remains unknown.

Given these considerations, this study was undertaken to address the following specific aims: (1) determine whether prior LPS exposure ('LPS preconditioning'; LPS-PC) alters subsequent LPS-mediated inflammatory/signaling events. Of note in this regard is a recent report that experimental Gram-negative sepsis, induced by cecal ligation and puncture, upregulates PT Toll-like receptor 4 (TLR4) levels.[11] This result, as well as 'endotoxin tolerance', could each alter subsequent renal sensitivity to LPS; (2) ascertain whether LPS-PC directly induces proximal tubular cell resistance to simulated ischemic (hypoxic) attack; (3) assess whether LPS injection increases PT levels of cholesterol, a stress-induced renal cytoprotectant;[12] and (4) probe whether such cholesterol alterations can potentially impact tubule cell resistance to injury as well as LPS signaling events.

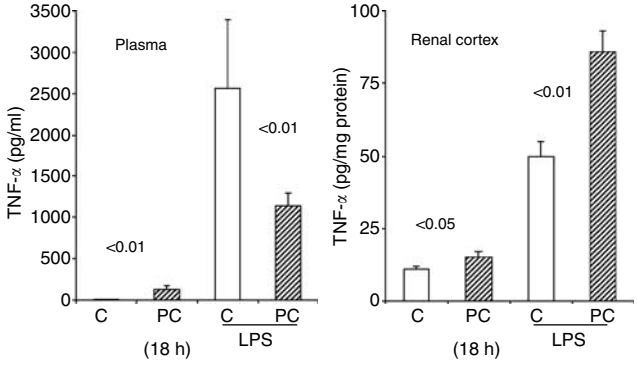

Figure 1 | TNF-α (protein) levels in plasma (left panel) and in renal cortex (right panel) in control mice, and in 18 h LPS-PC mice either under basal conditions or 2 h after an LPS challenge. Left panel: plasma TNF-α levels were basically undetectable in control mice (<2 pg/ml), and they were slightly elevated in the 18 h LPS-PC mice. At 2 h post LPS challenge, both the control and LPS-PC mice manifested striking TNF-α plasma increases compared to their baseline levels. However, the degree of increase was blunted by approximately two-third in the LPS-PC mice vs the LPS challenged controls. Hence, LPS-PC had induced a systemic 'LPS tolerance' state (as gauged by plasma TNF-α increases). Right panel: baseline renal cortical extract TNF-α levels were slightly elevated in the LPS-PC mice, compared to naive controls. Both groups responded to LPS with an acute increase in cortical TNF-α levels. However, unlike plasma levels, the LPS-PC mice manifested a statistically greater renal cortical TNF-α increase, compared to LPS-challenged naive controls. Thus, despite systemic LPS tolerance in the LPS-PC mice (based on plasma TNF-α levels), the LPS-PC mice manifested renal cortical hyper-responsiveness to repeat LPS injection.

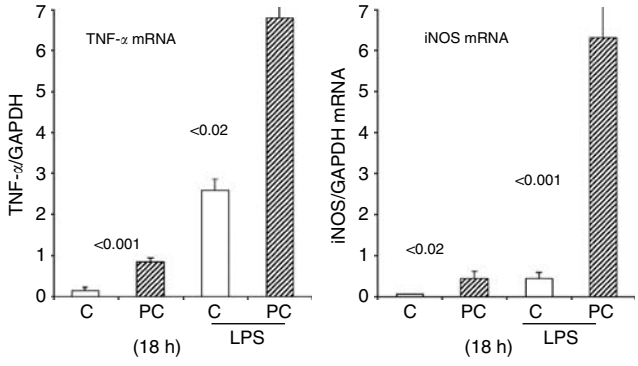

Figure 2 | Renal cortical TNF-α mRNA (left panel) and iNOS mRNA (right panel) values in control mice and 18 h LPS-PC mice either under basal conditions or 2 h after an acute LPS challenge/re-challenge. Left panel: at baseline, the 18 h LPS-PC mice had slightly higher TNF-α mRNA values vs controls. Both groups of mice responded to LPS injection with a dramatic increase in TNF-α mRNA; however, the degree of increase was markedly exaggerated (~2×) in the LPS-PC mice, indicating a renal cortical LPS hyper-responsive state. Right panel: at baseline, LPS-PC mice had slightly higher iNOS mRNA values than did controls. The control mice mounted only a modest increase in iNOS mRNA by 2 h post LPS injection. Conversely, a dramatic hyper-responsive state was observed in the LPS-PC mice, with iNOS mRNA values increasing ~6× more in the LPS-challenged preconditioned mice, vs their LPS-challenged naive controls. Thus, as with the TNF-α protein and TNF-α mRNA results, the iNOS mRNA results also indicated that preconditioning had induced a renal cortical hyper-responsive state.

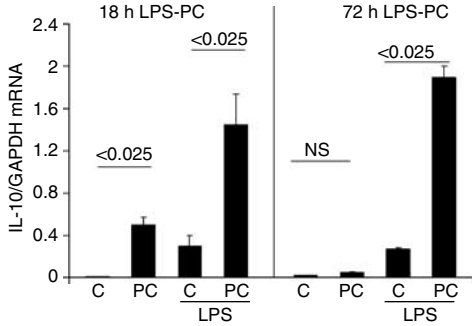

Figure 3 | Renal cortical IL-10 mRNA changes in control mice, 18 h LPS-PC mice (left panel) and 72 h LPS-PC mice (right panel) under basal conditions and 2 h after an acute LPS challenge/re-challenge. Renal cortical IL-10 mRNA values were elevated in the 18 h LPS-PC mice (vs controls). When either the 18 or 72 h preconditioned mice were re-challenged with LPS, far greater IL-10 mRNA increases resulted, vs those seen in LPS-challenged controls.

RESULTS

Eighteen hours of LPS-PC: renal tumor necrosis factor-α (protein) responses to subsequent LPS injection

As shown in Figure 1, mice that were subjected to 18 h of LPS-PC had slight, but significant, baseline elevations in both plasma and renal cortical tumor necrosis factor (TNF)-α levels (vs controls without LPS exposure). Both groups of mice responded to LPS with dramatic 2-h increases in plasma and renal cortical TNF-α levels. However, the plasma TNF-α increases were significantly blunted (~65%) in LPS-PC mice, vs LPS-challenged naive controls (implying systemic LPS tolerance). In contrast to plasma results, the LPS-PC mice manifested significantly greater renal cortical TNF-α increases upon LPS re-challenge, vs LPS-challenged naive controls. Thus, these experiments indicated an apparent dissociation of systemic (plasma) vs renal TNF-α responses to LPS. The 18 h LPS-PC mice had elevated blood urea nitrogens (67±5 mg/dl) vs controls (28±1 mg/dl; $P<0.01$). However, plasma creatinines remained unchanged (0.40±0.02 mg/dl for each group).

Eighteen hours LPS-PC: effects on renal TNF-α, inducible nitric oxide synthase, and interleukin-10 mRNA responses to LPS

As shown in Figures 2 and 3 (left panel), 18 h LPS-PC mice had slightly higher baseline renal cortical TNF-α mRNA, inducible nitric oxide synthase (iNOS) mRNA, and interleukin-10 (IL-10) mRNA values, vs controls. With a 2 h LPS re-challenge, the preconditioned mice developed 2–6× greater renal cortical TNF-α mRNA (Figure 2), iNOS mRNA (Figure 2), and IL-10 mRNA (Figure 3, left panel) increases than did the LPS-challenged naive controls, indicating an LPS hyper-responsive state.

Seventy-two hours LPS-PC: effects on TNF-α, iNOS, and IL-10 mRNA responses

The 72 h LPS-PC mice had slightly higher renal cortical TNF-α mRNA, iNOS, and IL-10 mRNAs, vs controls (Figure 3, right panel; Figure 4). The preconditioned mice retained

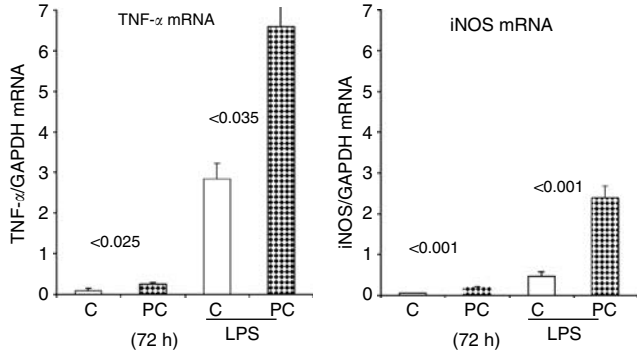

Figure 4 | Renal cortical TNF-α and iNOS mRNA levels in control mice and 72 h LPS-PC mice under basal conditions and 2 h following an LPS challenge. The 72 h LPS-PC mice had slightly elevated levels of TNF-α and iNOS mRNA at baseline, compared to values seen in the controls. By 2 h post LPS injection, the 72 h LPS-PC mice manifested hyper-responsiveness to LPS injection, as indicated by 2 × and 5 × greater TNF-α and iNOS mRNA increases, respectively, vs LPS-challenged naive controls. Thus, as with the 18 h LPS-PC experimental results, 72 h LPS-PC mice also demonstrated renal cortical hyper-responsiveness to LPS (as gauged by TNF-α and iNOS mRNA increases).

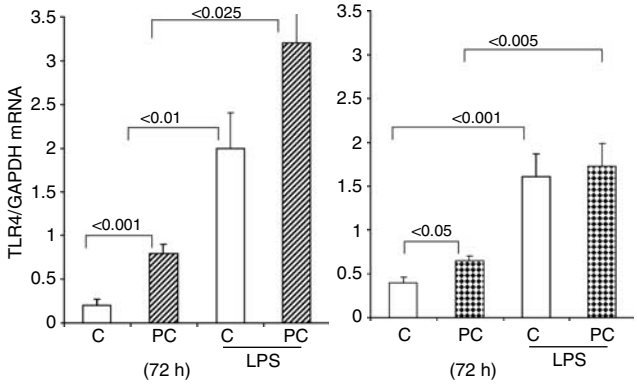

Figure 5 | Renal cortical TLR4 mRNA levels in control mice, in 18 h LPS-PC mice (left panel), and in 72 h LPS-PC mice (right panel) under basal conditions and 2 h following an acute LPS challenge. Left panel: after 18 h of LPS-PC, a significant increase in renal cortical TLR4 mRNA was observed, compared to control tissues. Both the control mice and the LPS-PC mice manifested brisk increases in TLR4 mRNA levels by 2 h post LPS injection. This acute response was moderately, but not significantly, higher in the LPS-PC group. Right panel: after 72 h of LPS-PC, slightly, but significantly, higher TLR4 mRNA values were seen in renal cortex, vs values seen in control cortex. Both the control and 72 h LPS-PC mice developed marked TLR4 mRNA increases by 2 h post LPS injection. That the LPS-PC mice manifested as great of a response to LPS as did the control mice indicated the absence of a renal cortical LPS-tolerant state.

hyper-responsiveness to LPS (2–6 × greater increases in TNF-α, iNOS, and IL-10 mRNAs, vs LPS-challenged controls; assessed 2 h post LPS challenge).

The 72 h PS-PC mice had a two-third blunted increase in plasma TNF-α levels in response to a 2 h LPS challenge, compared to LPS-challenged controls (1103 vs 3392 ± 280 pg/ml; $P < 0.015$). This again implies systemic LPS tolerance at a time of renal cortical hyper-responsiveness of TNF-α, iNOS, and IL-10 mRNAs.

Renal cortical monocyte chemotactic protein-1 mRNA responses

Baseline monocyte chemotactic protein-1 (MCP-1) mRNA values were dramatically increased in both 18 and 72 h LPS-PC mice (controls, 0.1 ± 0.01; 18 h LPS-PC, 1.2 ± 0.2; 72 h LPS-PC, 1.4 ± 0.3; $P < 0.01$ vs controls). However, after the 2 h LPS challenge, no preferential MCP-1 mRNA increase was observed in the LPS-PC vs the control group (2.2 ± 0.4, 2.3 ± 0.3, and 2.5 ± 0.4; controls, 18 and 72 h LPS-PC, respectively). No azotemia existed in the 72 h of LPS-PC mice (blood urea nitrogen, 28 ± 3 mg/dl; creatinine, 0.4 ± 0.02 mg/dl, nonsignificant vs controls).

Renal cortical TLR4 mRNA and protein: impact of LPS-PC

mRNA. Significant TLR4 mRNA increases were seen in both the 18 h LPS-PC mice (Figure 5, left panel) and 72 h LPS-PC mice (Figure 5, right panel), vs controls. Both sets of mice manifested marked TLR4 mRNA increases by 2 h post LPS challenge/re-challenge (Figure 5). Preconditioning did not diminish the LPS-initiated TLR4 mRNA increases: if anything, heightened sensitivity was suggested in the 18 h LPS-PC animals.

Western blotting. As previously described and depicted,[13] TLR4 appears as two closely related bands seen at ~90 kDa. LPS-PC did not increase TLR4 protein levels in renal cortex (controls, 2198 ± 212 density units; 18 h LPS-PC, 1715 ± 154 density units) or in isolated tubules (controls, 2167 ± 76 density units; 18 h LPS-PC, 2141 ± 172 density units). When assessed at 72 h post LPS injection, no difference in TLR4 protein levels was observed (controls, 2072 ± 216; LPS-PC, 2031 ± 145; nonsignificant). Thus, although the cecal ligation and puncture sepsis model may increase TLR4 protein levels,[11] pure LPS injection need not produce this result.

LPS-PC: impact on proximal tubular resistance to hypoxic injury

Eighteen hours LPS-PC. As shown in Figure 6, isolated PTs harvested from control and 18 h post LPS-PC mice demonstrated no significant difference in viability under control (oxygenated) conditions. Exposure to either 7.5 min or 10 min of hypoxia, followed by reoxygenation, caused stepwise increases in tubular cell death (% lactate dehydrogenase (LDH) release) in control tubules. PTs obtained from 18 h LPS-PC mice manifested significant protection against both the 7.5 and 10 min hypoxic challenge.

Seventy-two hours LPS PC. Tubules harvested from control mice and 72 h LPS-PC mice were subjected to 10 min hypoxic-reoxygenation injury. Again, tubules from LPS-PC mice manifested partial resistance to lethal hypoxic damage (Figure 6; $P < 0.015$).

Assessment of TNF-α levels in isolated tubules

TNF-α levels were slightly elevated in isolated tubules prepared from 18 h LPS-PC mice vs controls (Figure 7). When tubules were harvested from control and LPS-PC mice

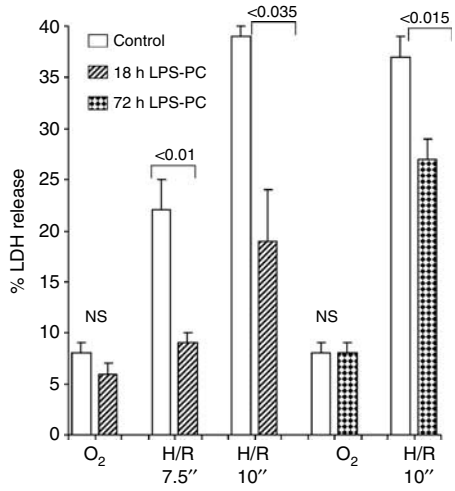

Figure 6 | Isolated PT susceptibility to hypoxic-reoxygenation (H/R) injury. Isolated PTs, prepared from control mice, 18 h LPS-PC mice, and 72 h LPS-PC mice were subjected to either control oxygenated (O_2) incubations $\times$ 15 min or to either 7.5 or 10 min of hypoxia, followed by 7.5 or 5 min reoxygenation, respectively. No significant difference in tubule viability was observed between control tubules or LPS-PC tubules under control (oxygenated) incubation conditions. However, tubules obtained from LPS-PC mice manifested consistent protection against H/R injury whether the challenge was 7.5 or 10 min of hypoxia, or whether the challenge was imposed at either 18 or 72 h of preconditioning. Cell injury was assessed by % LDH release.

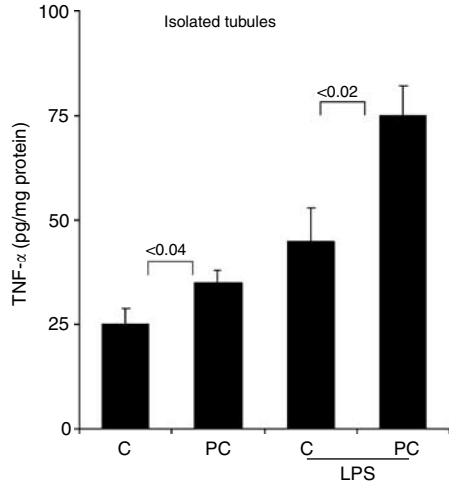

Figure 7 | TNF-α levels in isolated PTs obtained from control and 18 h LPS-PC mice without or with a 2 h LPS challenge. Baseline TNF-α levels were slightly higher in tubules harvested from the 18 h LPS-PC mice vs control mice. Both the preconditioned mice and control mice manifested TNF-α increases in response to the 2 h LPS challenge/re-challenge. The degree of increase was greater in the LPS-PC mice than in the controls, confirming hyper-responsiveness to LPS in the former group. Thus, these isolated tubule results were consistent with the observations obtained in whole renal cortex.

2 h post LPS challenge/re-challenge, a significantly greater TNF-α increase was observed in the LPS-PC group. Thus, these isolated tubule data confirm that the preferential renal cortical TNF-α increases seen in LPS-PC mice upon LPS re-challenge (Figure 1, data) reflected, at least in part, PT (vs circulating cytokine/inflammatory cell) events.

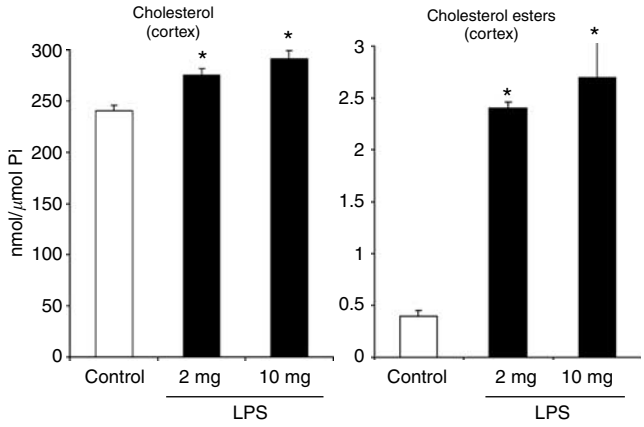

Figure 8 | Renal cortical free cholesterol (left panel) and cholesterol ester (right panel) levels in control mice and mice that had undergone 18 h of LPS-PC with either 2 or 10 mg/kg LPS. Statistically significant increases in both free and esterified cholesterol levels were observed in the LPS-PC groups, and in a dose-dependent manner (*$P \leqslant 0.02$ vs control values).

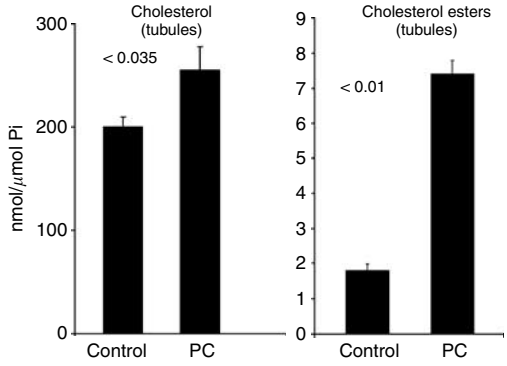

Figure 9 | Free cholesterol (left panel) and cholesterol ester (right panel) levels in control (C) tubules and in tubules harvested from 18 h LPS-PC mice. Both free and esterified cholesterol levels were significantly elevated in tubules from PC mice, indicating that the elevations seen in renal cortex (Figure 8) reflected, at least in part, proximal tubular cell events.

LPS-PC: renal cholesterol levels and tubule resistance to cholesterol attack

Cortical cholesterol assessments. Each of the employed LPS-PC protocols caused significant increases in renal cortical cholesterol/cholesterol ester content (Figure 8). At 72 h post 2 mg/kg LPS (not depicted), comparable cholesterol elevations were seen (LPS-PC, 285 ± 12 nmol/μmol; controls, 248 ± 2 nmol/μmol inorganic phosphate; $P < 0.015$).

Isolated tubule cholesterol levels. Isolated tubules from LPS-PC mice also had significantly higher cholesterol levels than did control tubules (Figure 9), recapitulating the whole renal cortex cholesterol results.

Resistance to cholesterol attack. As shown in Figure 10, PTs harvested from LPS-PC mice showed significant cytoresistance to direct plasma membrane cholesterol attack, induced by either cholesterol esterase (CE) or cholesterol oxidase ($\sim$35% reductions in lethal cell injury, LDH release, vs identically challenged control tubules). Thus, as with the

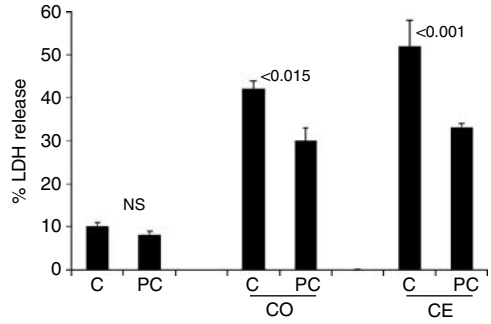

Figure 10 | Isolated tubule responses to direct plasma membrane cholesterol attack with either cholesterol oxidase (CO) or CE. With either a cholesterol oxidase or a CE challenge, tubules harvested from 18 h LPS-PC mice demonstrated relative resistance to injury (LDH release), compared to results observed in control tubules. Under unchallenged conditions (far left two bars), no difference in viability between control (C) and preconditioned mice (PC) was observed.

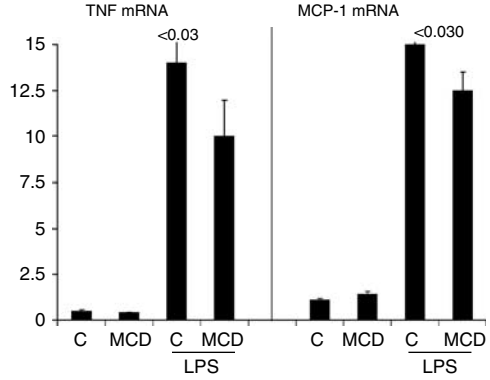

Figure 11 | HK-2 cell TNF-α and MCP-1 mRNA responses to LPS with and without acute cholesterol reductions. The cholesterol stripping agent, MCD, did not impact levels of either mRNA under basal conditions. However, MCD pretreatment significantly blunted the degree of TNF-α and MCP-1 mRNA increases that resulted from a 3 h acute LPS exposure.

above described hypoxia results, LPS-PC induced a direct PT cytoresistant state, with membrane cholesterol resistance likely being at least partially involved.

Impact of cell cholesterol on LPS-mediated signaling

Methylcyclodextrin/cholesterol extraction experiments. A 1-h methylcyclodextrin (MCD) protocol was employed to reduce HK-2 cell cholesterol by ~30% (Zager *et al.*[12]), and then its impact on LPS signaling was assessed. MCD did not independently alter HK-2 TNF-α or MCP-1 mRNA levels (Figure 11). LPS evoked massive TNF-α and MCP-1 mRNA increases. MCD pre-treatment significantly blunted these LPS-TNF-α/MCP-1 mRNA responses.

A2C membrane fluidity experiment. Increased membrane cholesterol decreases membrane fluidity.[12] Hence, the impact of membrane fluidity on LPS signaling was also assessed. Addition of a membrane fluidizing reagent (A2C; Zager *et al.*[12]) to HK-2 cells did not independently alter TNF-α/MCP-1 mRNA levels (Figure 12). However, A2C significantly blunted LPS-mediated TNF-α and MCP-1 mRNA increments.

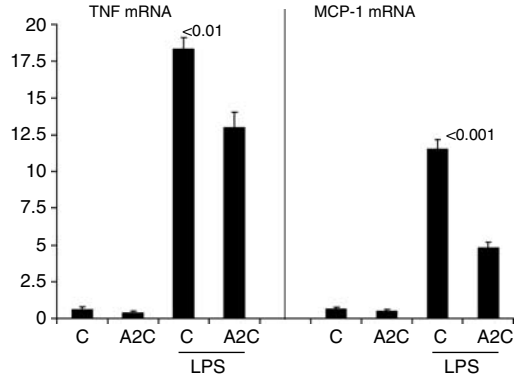

Figure 12 | HK-2 cell TNF-α and MCP-1 mRNA responses to LPS with and without the addition of a membrane-fluidizing agent, A2C. A2C did not significantly alter basal TNF-α or MCP-1 mRNA levels. However, A2C significantly blunted both mRNA responses to LPS addition. Thus, both changes in membrane cholesterol (Figure 11, results) and cholesterol-associated changes in membrane fluidity significantly impact LPS inflammatory signaling events.

Thus, both cholesterol reduction (which increases membrane fluidity) and a membrane-fluidizing reagent (A2C) induced comparable reductions in LPS-driven TNF-α and MCP-1 mRNA responses.

DISCUSSION

Endotoxin tolerance is classically defined as a reduced capacity of previously exposed animals to respond to a second LPS challenge. However, this 'hypo-responsiveness' is not necessarily global in its expression, given that selective signaling pathways may be downregulated while leaving others intact (e.g., Fan and Cook,[1] Ziegler-Heitbrock,[2] Cross,[3] Learn *et al.*,[4] Ziegler-Heitbrock *et al.*[5]). Characterization of 'LPS tolerance' is further complicated by the fact that tissue-specific differences in its expression may exist. Thus, whereas LPS-preconditioned macrophages may downregulate production of selected cytokines or chemokines, it cannot be assumed that the same pattern will be observed in other cell types or in whole tissues.[1–5] Given these considerations, the present study was undertaken to determine the impact of LPS-PC on renal, and proximal tubular, responsiveness to selected components within the LPS signaling cascade. Toward this goal, a broad range of reactants, induced via differing 'downstream' TLR4 pathways,[14,15] were screened (TNF-α, a pro-inflammatory cytokine; IL-10, an anti-inflammatory cytokine; MCP-1, a chemokine; and iNOS, producing the gaseous oxidant, NO).

That the employed LPS-PC protocols did, in fact, induce systemic LPS tolerance was confirmed by the results of plasma TNF-α assays. LPS injection into naive controls evoked massive plasma TNF-α increases, rising from <2 to ~3000 pg/ml (determined at 2 h post LPS injection). This response was reduced by approximately two-third in both the 18 and 72 h LPS-PC mice, with peak TNF-α levels of ~1100 pg/ml being observed. Paradoxically, the kidney failed to participate in this LPS tolerant state: rather than manifesting suppressions in renal cortical TNF-α concentrations

upon LPS re-challenge, the 2 h post LPS injection renal cortical values were approximately twice as high in LPS-PC mice, compared to LPS challenged naive controls. Renal cortical TNF-α mRNA assessments underscored that the LPS-PC mice did, in fact, paradoxically hyper-respond to LPS. As shown in Figure 2, the TNF-α mRNA increases, assessed 2 h post LPS re-challenge, were ~2–3 times greater in the 18 h LPS-PC mice, vs the naive, LPS challenged, controls. To explore whether this hyper-responsiveness was simply a transient phenomenon, TNF-α mRNA levels were also assessed in 72 h LPS-PC mice. Once again, the LPS-PC mice manifested two- to threefold greater renal cortical TNF-α mRNA increases following LPS injection, compared to LPS challenged controls. Notably, TNF-α assays performed on isolated tubules obtained from LPS-PC and control mice following an acute LPS challenge confirmed ~2× higher values in the former group. The significance of this finding is that it confirms that the renal cortical results reflected, at least in part, proximal tubular cell events (as opposed to glomerular, vascular changes; infiltrating inflammatory cells, or circulating cytokine levels). Finally, it is notable that this renal cortical hyper-responsive state was not restricted to TNF-α, given that LPS-mediated iNOS mRNA and IL-10 mRNA increases were ~three- to sixfold greater in LPS-PC mice, vs their equivalent controls. In contrast, LPS-mediated MCP-1 mRNA increases were not enhanced in LPS-PC animals. This simply underscores that although LPS-PC may induce renal LPS 'hyper-responsiveness', this state need not affect all components of the LPS-TLR4 inflammatory cascade.

El-Achkar *et al.*[11] recently reported increased PT TLR4 expression in the cecal ligation and puncture model of Gram-negative sepsis. If purified LPS injection were to induce the same result, it might explain the observed increase in renal LPS responsiveness in preconditioned animals. However, the available data seemingly rule out this possibility, given that renal cortical/isolated tubule Western blots showed no TLR4 protein increases in 18 or 72 h LPS-PC animals. In contrast to TLR4 protein, TLR4 mRNA levels were consistently increased by LPS treatment. For example, ~4× and 2× increases were observed at 18 and 72 h post LPS injection, respectively. Furthermore, at 2 h post LPS re-challenge, the preconditioned kidneys mounted further sharp (~2×) TLR4 mRNA increases, equaling or exceeding those seen in LPS-challenged controls. Indeed, the pronounced LPS-mediated TLR4 mRNA increases in preconditioned mice seem antithetical to the presence of a renal 'LPS tolerant state', given that decreased responsiveness would be expected. Of note, we recently reported that renal TLR4 mRNA levels abruptly increase in response to diverse forms of acute renal damage (cisplatin, myohemoglobinuria, ischemia–reperfusion; Zager *et al.*[13]). These latter findings help place the current observation of LPS-induced TLR4 mRNA elevations in a new perspective, that is, they may simply represent part of a generic renal 'stress response', rather than reflecting an LPS-specific change, *per se*.

As previously noted, there has been debate as to whether LPS-PC protects against ischemia–reperfusion-induced acute renal failure,[8,9] and if so, whether that protection stems from altered cytokine/chemokine profiles[8] vs direct PT events. The present isolated tubule experiments indicate that direct PT cytoresistance against adenosine trisphosphate depletion injury does, indeed, occur, based on observations that LPS-PC tubules manifested resistance to hypoxic attack. To our knowledge, this is the first demonstration that LPS-PC can confer protection directly at the proximal tubular cell level (vs being secondary to systemic inflammatory or hemodynamic events). Indeed, this observation suggests a seeming paradox: that renal tubular cytoresistance can be expressed, or can coexist, with a renal LPS hyper-sensitivity state (as denoted by the present TNF-α/iNOS/IL-10 data). It is notable that we have documented this same pathophysiologic profile, that is, increased renal LPS sensitivity in the setting of acquired cytoresistance, in diverse structural models of acute renal failure (e.g., rhabdomyolysis; cisplatin; ischemia–reperfusion; obstructive nephropathy; Zager *et al.*[16–18]). Thus, it would appear that the present results, obtained with an endotoxemic/hemodynamic model[19–21] of renal injury, are reflective of a much broader biologic principle than simply 'endotoxin preconditioning', that is, that diverse models of acute renal stress can induce proximal tubular cytoresistance, and that the latter can coexist with a potentially 'injury-amplifying' (TNF-α/iNOS) hyper-inflammatory state.

Finally, it is tempting to speculate as to whether a common pathway gives rise to, or contributes to, both post-injury LPS hyper-responsiveness and the tubular cytoresistance phenomenon. Although it is premature to draw firm conclusions in this regard, renal cortical cholesterol accumulation could be one such shared mechanism. We have previously documented that in the aftermath of diverse forms of nephrotoxic, ischemic, and obstructive renal injury, renal cortical/PT cholesterol levels rise, and that the cholesterol increases help confer cellular resistance to subsequent hypoxic and toxic attack.[12,22–31] This mechanistic association has been deduced from the following observations: preventing cholesterol accumulation (with statins), normalizing cholesterol in post-injured cytoresistant tubules (with MCD), or altering cholesterol integrity, each predisposes to tubular injury and/or reverses the cytoresistant state.[12,22–31] We now confirm that endotoxemia, like other renal insults, also increases proximal tubular cholesterol content. That direct plasma membrane resistance to cholesterol attack results (decreased LDH release in response to CE or oxidase) further supports this mechanistic link. It is noteworthy that plasma membrane cholesterol may alter non-LPS-driven cell signaling events.[32–35] Therefore, we have tested, for the first time, whether changes in tubular cell cholesterol levels can alter LPS responsiveness. Preliminary studies indicate that the answer is yes. Using an MCD protocol that reduces HK-2 cell cholesterol by ~30%,[12] an approximate 30% reduction in LPS-mediated TNF-α and MCP-1 mRNA generation resulted. Plasma membrane cholesterol accumulation decreases

membrane fluidity.[12] Hence, we assessed whether membrane fluidity might also impact HK-2 cell LPS responsiveness. Again the answer was yes: the membrane-fluidizing reagent A2C blunted the LPS-mediated TNFα/MCP-1 mRNA increases, again by ~30%. As A2C and MCD have each been shown to decrease cytoresistance,[12] it seems plausible that injury-induced cholesterol increases, with decreased membrane fluidity, could contribute both to cytoresistance as well as the post-injury LPS hyper-responsive state. Indeed, cholesterol loading could help to explain why LPS-PC, as induced in this study, failed to initiate a classic renal 'endotoxin tolerant' state. Further exploration of this intriguing 'unifying cholesterol hypothesis' is required, and this remains an active area of investigation within this laboratory.

MATERIALS AND METHODS

Animal utilization

All *in vivo* experiments were conducted with male CD-1 mice (25–30 g), obtained from Charles River Laboratories (Wilmington, MA, USA). They were maintained under routine vivarium conditions with free food and water access. Tail vein (intravenous) injections were performed with 26 G needles after placing the mice in restraining tubes. Kidneys resections were performed through midline abdominal incisions under pentobarbital (40–50 mg/kg) anesthesia. Kidneys were immediately iced, the cortices were dissected, and subjected to either protein or total RNA extraction.[27] Terminal heparinized blood samples were drawn from the inferior vena cava.

In vivo experiments

LPS-PC: effect on renal TNF-α, iNOS, IL-10, MCP-1, and TLR4 expression. *Eighteen hours of LPS-PC:* Mice were injected with *Escherichia coli* LPS (10 mg/kg; 0111:B4; L-2630; Sigma Chemicals (St Louis, MO, USA); in ~80 μl saline; *n*, 12) or its saline vehicle (control group; *n*, 12). Eighteen hours later, the mice received a second tail vein injection of either LPS (2 mg/kg) or vehicle, creating the following four groups (*n*, 6 per group):

Group (1): control mice (saline injection at baseline, and again 18 h later);
Group (2): LPS-PC mice (baseline LPS; followed by saline injection 18 h later);
Group (3): control mice/LPS challenge (saline at baseline, followed by LPS 18 h later);
Group (4): LPS-PC mice/LPS challenge (baseline LPS; followed by LPS 18 h later).

Two hours following the second intravenous injection, plasma and kidney samples were obtained, as noted above. The plasma samples were analyzed for blood urea nitrogen, creatinine, and TNF-α concentrations (by enzyme-linked immunosorbent assay; R&D Systems, Minneapolis, MN, USA; Zager *et al.*[16]). Tissues from one kidney underwent protein extraction and were analyzed for TNF-α (by enzyme-linked immunosorbent assay; Zager *et al.*[16]). The contralateral cortical tissue samples underwent RNA extraction. Analyses for TNF-α, iNOS, MCP-1, IL-10, and TLR4 mRNAs were performed by competitive reverse transcriptase-polymerase chain reactions as described previously.[13,16–18] Simultaneously obtained glyceraldehyde-3-phosphate dehydrogenase (GAPDH) product served as a housekeeping gene (individual results being expressed as a ratio to the simultaneously obtained GAPDH product).

Seventy-two hour of LPS-PC: The above experiment was repeated with the following exceptions: (i) a 72 h period was allowed following initial LPS injection; and (ii) the preconditioning LPS dosage was 2 mg/kg (as not all mice could survive for 72 h following 10 mg/kg LPS injection).

TLR4 Western blot analyses. Renal cortical protein extracts, obtained from the following groups of mice, underwent TLR4 Western blot analysis:[13] six control kidneys; six kidneys obtained 18 h post 10 mg/kg LPS injection; six kidneys obtained 72 h post 2 mg/kg LPS injection; and six time matched (72 h) controls. To gain PT-specific results, TLR4 was probed in isolated PTs obtained from 18 h LPS-PC mice (*n*, 4) and control mice (*n*, 4), as described below. The results were expressed as densitometry units.[13]

Isolated PT experiments

Tubular susceptibility to hypoxic injury: impact of LPS-PC. *Eighteen hours of LPS-PC:* Mice were injected with either LPS (10 mg/kg intravenous) or saline vehicle (*n*, 4 each). Eighteen hours later, they were anesthetized with pentobarbital, a blood sample was obtained for blood urea nitrogen/creatinine determination, and then the kidneys were resected and iced. Cortical PTs were isolated by collagenase digestion/differential centrifugation,[12] and placed in experimentation buffer.[12] One control and one LPS-exposed mouse were studied in pairs on any given day. The PT preparations from each mouse were each divided into four equal aliquots, maintained at 37°C in 10 ml Erlenmeyer flasks, and subjected to one of the following conditions: (1) control (oxygenated) incubation (95% O_2/5% CO_2) × 15 min; (2) 7.5 min of hypoxia (95% N_2/5% CO_2), followed by 7.5 min of reoxygenation; (3) 10 min of hypoxia + 5 min reoxygenation; and (4) a second control incubation. Lethal cell injury was assessed by % LDH release.[12]

Seventy-two hours of LPS-PC: Four mice were injected with 2 mg/kg of LPS or vehicle. PTs were harvested 72 h later, and subjected to 10 min of hypoxia + 5 min reoxygenation, or 15 min control (oxygenated) incubation. LDH release was then assessed.

Assessments of tubule TNF-α cytokine and TLR4 protein levels. PTs were prepared from the following groups of mice (*n*, 4 per group): (1) control tubules; (2) LPS-PC mice (injected 18 h earlier with 10 mg/kg); (3) non-conditioned mice 2 h post LPS injection (2 mg/kg); and (4) LPS-PC mice 2 h after re-challenging with LPS. The tubules were kept at isolation temperature (4°C), protein extracts were prepared, and assayed for TNF-α. To gauge PT TLR4 content, control and LPS-PC tubule protein extracts were probed for TLR4 by Western blot, as above.[13]

Cholesterol experiments

Renal cortical/PT cholesterol assessments. Five groups (*n*, 4 each) of mice were created as follows: (Groups 1 and 2): 2 or 10 mg/kg LPS exposure × 18 h; (Group 3): 2 mg/kg LPS exposure × 72 h; (Groups 4 and 5): 18 or 72 h time matched controls. At the appropriate times, the kidneys were removed, the cortices were dissected, subjected to lipid extraction, and assayed for free and esterified cholesterol by gas chromatography.[23] Values were expressed as nmol/μmol phospholipid phosphate (Pi).[23] To confirm that the whole cortex results reflected, at least in part, PT events, cholesterol/cholesterol ester levels were also assessed in isolated tubules obtained from five control mice and five mice preconditioned 18 h earlier with 10 mg/kg LPS.

Proximal tubular resistance to direct cholesterol attack. To ascertain whether tubular cholesterol loading alters cellular resistance to direct cholesterol attack, five sets of isolated tubules, obtained from five normal mice and five 18 h LPS-PC mice, were prepared and divided into four aliquots: (1) control incubation; (2) exposure to cholesterol oxidase (2 U/ml; Zager[22]); exposure to CE (1 U/ml; Zager[22]); or (4) second control incubation. After 30 min, % LDH release was assessed.

HK-2 cell cholesterol content as a potential modifier of LPS responsiveness. HK-2 proximal tubular cells were cultured in 16 T25 flasks.[12] Four groups (four flasks per group) were created: (1) control incubation; (2) ~30% cholesterol reduction, induced with MCD (a cholesterol 'stripper'; 10 mM × 1 h followed by washout; Zager *et al.*[12]); (3) LPS challenge (10 μg/ml × 3 h); (4) LPS challenge × 3 h in MCD pretreated cells (note: all cells underwent the same washing procedures as used to remove MCD). At the end of the 4 h experiment, TNF-α, MCP-1, and GAPDH mRNAs were assessed.[16,17]

Membrane fluidity as a potential modifier of LPS responsiveness. Because increases in cholesterol reduce plasma membrane fluidity,[12] the impact of membrane fluidity on TNF-α/MCP-1 mRNA responsiveness to LPS was assessed. Four experimental groups (*n*, 4 each) of HK-2 cells in T25 flasks were established: (1) control incubation × 4 h; (2) incubation × 4 h with 100 μM A2C (Zager *et al.*,[12] a fluidizing reagent); (3) 1 h control incubation + 3 h LPS exposure (10 μg/ml); and (4) A2C × 1 h followed by 3 h of combined A2C + LPS exposure. At the end of the 4 h experiments, TNF-α, MCP-1, and GAPDH mRNA levels were assessed.

Calculations and statistics

All values are expressed as means ± 1 s.e.m. Statistical comparisons were performed by paired or unpaired Student's *t*-test. mRNA results were expressed as a ratio to simultaneously determined GAPDH product.

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